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WHEAT BRAN - PROPITIOUS SUBSTRATE FOR VANILLIN PRODUCTION BY *PSEUDOMONAS FLUORESCENCE*

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ABSTRACT

Natural flavor production by direct extraction from botanic sources can no longer satisfy the large market demand because of low concentrations of desired product in plants, Alternative natural sources for flavors production are needed and biotechnology is, by far, the most attractive field of exploration. In general, the advantages of biotechnological approaches are mild reaction conditions, high regio- and enantioselectivity leading to only one product isomer, no formation of toxic wastes and thus fewer environmental problems. An attempt was made in the present study to produce vanillin from ferulic acid recovered from wheat bran hydrolyzates by *Pseudomonas fluorescens*. Meat samples were collected and processed to isolate *P. fluorescens* and maintained on LB agar.Wheat bran was collected and processed to obtain ferulic acid by solvent extraction method.Fermentation of wheat bran with 0.05% ferulic acid as inducer was performed using *P. fluorescens* for the production of vanillin.After 3 days of fermentation, the production of vanillin in the medium was estimated by HPLC.Samples were run in HPLC along with standard vanillin. Sample showed a single peak with retention time 2.902minutes with its area (%) 92.74 respectively. The standard vanillin showed a single peak with retention time 2.888minutes with its area (%) 100 correspondingly.The peak at 2.888-2.902 (retention time) in standard vanillin corresponds to the sample from fermented medium, whose percentage purity is 99%. This proved the presence of vanillin in the fermentation medium.The amount of vanillin produced was 12gms/litre.

KEYWORDS: Ferulic acid, Wheat bran, Vanillic acid,Vanillin. ***Pseudomonas fluorescence.***

INTRODUCTION

Vanillin or 4-Hydroxy-3-methoxy-benzaldehyde is the world's principal flavouring compound which is used extensively in the food industry, perfumery, and beverage besides being applied in the pharmaceutical industry. Vanillin can be produced synthetically or naturally from vanilla beans[1] Its major component, vanillin is the key compound contributing to vanilla characteristic aroma and is the second largest aroma produced in the world. At present,majority (≥80%) of vanillin supplied to the world market is chemically synthesized from a petroleum-based raw material "guaiacol" with less than 12–14% produced from lignin-containing waste produced by the sulfite pulping process.

Production of vanillin by microbial or enzymatic conversion of natural precursors such as ferulic acid, vanillic acid, glucose and eugenol has been investigated. It is a highly sought-after flavor compound and is widely used in the food, confectionery, perfumery, cosmetics and pharmaceutical industries. High demand for natural flavors from consumers has triggered research in natural vanillin production from natural resources through microbial biotransformation.[2]

Biotechnology has the potential to generate through biotransformation carried out by microorganisms and their enzymatic systems.[3] Ferulic acid, a widely-known natural phenolic compound from lignin degradation by fungi and bacteria was deeply studied as vanillin precursor.. Common agricultural waste residues such as cereal bran, rice bran, sugar beet pulp, and wheat straw are rich sources of ferulic acid (FA), which is an important precursor of microbially synthesized vanillin and can be exploited as raw material for transformation to vanillin by microorganisms.[4]

The conversion of ferulic acid to vanillin via microbial routes is one of the most intensely studied biotransformation and also the most promising process for commercial production of biovanillin. Moreover ferulic acid may be the major suitable candidate for biovanillin production, being least toxic of all the investigated precursors. The biotransformation of FA to vanillin was widely investigated in several microorganisms, including Gram-negative bacteria of the *Pseudomonas* genus, actinomycetes of the genera *Amycolatopsis* and *Streptomyces*[5], Gram-positive bacteria, such as *Bacillus subtilis* and *Rhodococcus sp.*[6] and

the basidiomycete fungi, such as *Pycnoporus cinnabarinus*.[7] *Polyporous versicolor* and *Fomes fomentarius* were proposed for the bioconversion of ferulic acid to vanillin.

Several bacteria belonging to different genera are able to metabolize ferulic acid as sole carbon source leading to the production of vanillin, vanillic acid and protocatechuic acid as catabolic intermediates the most interesting of which being *Pseudomonas fluorescens*. Bacteria belonging to genera *Pseudomonas* sp. were found to be interesting candidates for their capability of converting ferulic acid as sole carbon source to vanillin.

Hence in the current investigation an attempt was made to evaluate the possibility of producing vanillin from ferulic acid recovered from wheat bran hydrolyzates by using cells of *Pseudomonas fluorescens*.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PROCESSING

Meat samples were collected in clean, dry and sterile polythene bags and processed on the surface of sterile Cetrimide – Fusidine – Cephaloridine (CFC) agar plates for enumeration of ***Pseudomonas sp.*** All the plates were incubated at 30^{o}C for 24 - 48 hours .The isolated organisms were identified as per the Bergey's manual of systemic bacteriology, based on microscopic morphology, biochemical characteristics and pigment production.

PRODUCTION OF VANILLIN BY *PSEUDOMONAS FLUORESCENS*

Collection of raw material

Wheat bran was collected from nearby milling industry using sterile polythene bags.

Extraction of ferulic acid from wheat bran[8]

Ferulic acid was extracted from wheat bran by solvent extraction method**.** Finely grounded bran was refluxed with petroleum ether for 30 min. The solution was filtered and the residue was treated with 1M NaOH with a solid to liquid ratio of 1:10 (g/ml) for 4h at 40^{o}C.The solution was filtered and the filtrate was neutralized with 6M HCl to pH 6.0.The solution was concentrated and 3 volumes of ethanol was added to precipate hemicellulose.The solution was filtered and ethanol was evaporated. 0.1M H_2SO_4 was added to precipate lignin at pH 1.5. The filtrate was extracted with 3 volumes of chloroform and methanol.The methanol layer was dried under vaccum and the ferulic acid was obtained.

Bioconversion of ferulic acid to vanillin[9]

Pure cultures of ***Pseudomonas fluorescens*** was maintained on Luria Bertani (LB) agar and incubated at 37^{o}C for 24 - 48 hours. Colonies of ***P.fluorescens*** was then transferred to 100ml of LB broth (Luria Bertani) and incubated at 37^{o}C for 24-48 hours.1% inoculum was added to the fermentation broth consisting of (g/100ml): 15g of wheat bran and 0.05% of ferulic acid as inducer for biotransformation.(pH – 5.6). The fermentation broth was incubated at 37^{o} C for 48-72 hours at 180 rpm under shaking conditions. After 72 hours of biotransformation vanillin was estimated from the fermentation medium by HPLC Analysis.

HPLC analysis of vanillin produced in the fermentation medium

The fermented mass was centrifuged after biotransformation to remove the bran particles. The supernatant was acidified to pH 2 using 6N HCl. Phenolic compounds were extracted thrice with a equal volumes of ethyl acetate. Then the supernatant was used for the estimation of vanillin by HPLC.The production of vanillin in the fermentation broth was analysed with the HPLC system. The separation was carried out using C18 column, 4.6 x 250mm(5µ) The mobile phase consisted of acetonitrile in water in the ratio of 90:10 and the flow rate was 1.0ml/min. The sample was filtered through 0.22µm membrane and 20µl was directly injected into the column of HPLC and the products were detected at 230nm.The separation was carried out simultaneously for standard vanillin.

RESULTS

Samples of meat were collected and processed for the isolation of *Pseudomonas fluorescens.* Based on the microscopic morphology biochemical characteristics, pigment production test and extracellular enzyme production, the isolate was identified as *Pseudomonas fluorescens.*

Biosynthesis of vanillin by *Pseudomonas fluorescens*

Extraction of ferulic acid from wheat bran

Solvent extraction method was used for the extraction of ferulic acid from wheat bran. The extracted ferulic acid was granulated and appeared white in colour. It was dried in a vacuum evaporator to get finely powdered ferulic acid.

Bioconversion of ferulic acid to vanillin

Pseudomonas fluorescens was inoculated into the fermentation broth containing wheat bran as a substrate with 0.05% ferulic acid as inducer. After a suitable period of incubation presence of fluorescent green pigment indicated the luxurious growth of *P.fluorescens* while

no fluorescent green pigment was observed in the control flasks (without inoculum) respectively.

HPLC analysis of vanillin produced in the fermentation medium

The concentration of vanillin produced after 3 days of fermentation was estimated by HPLC. The supernatant obtained after centrifugation of the fermented mass was used in the HPLC analysis. The estimation of standard vanillin was also performed. Samples from the fermented medium showed a single peak with a retention time 2.902minutes with area 92.74%. Standard vanillin showed a single peak with retention time 2.888minutes with area 100%. The peak with retention time 2.90minutes observed in the fermented sample corresponds to the peak with the retention time 2.888minutes of standard vanillin whose percentage of purity is 99%. The amount of vanillin was produced to found to be **12gms/litre.** This indicates the production of vanillin by the bioconversion activity of *P.fluorescens* using ferulic acid as inducer in the fermentation medium.

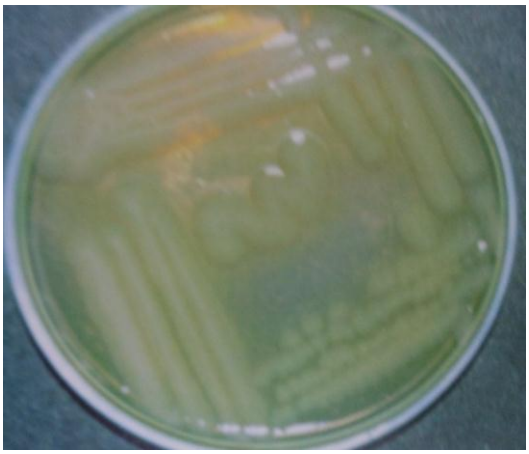


Plate 1: Pigmentation In King's B Medium.

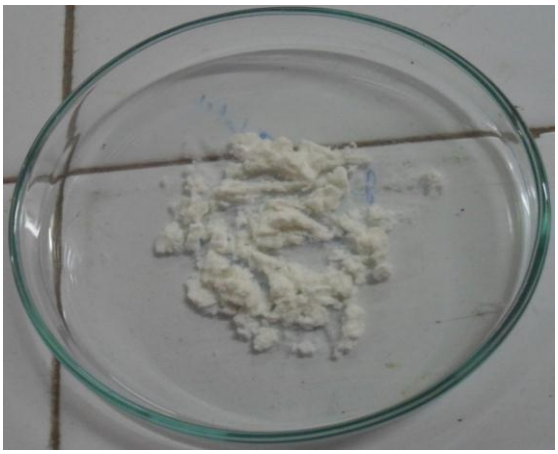


Plate 2: Extracted Ferulic Acid

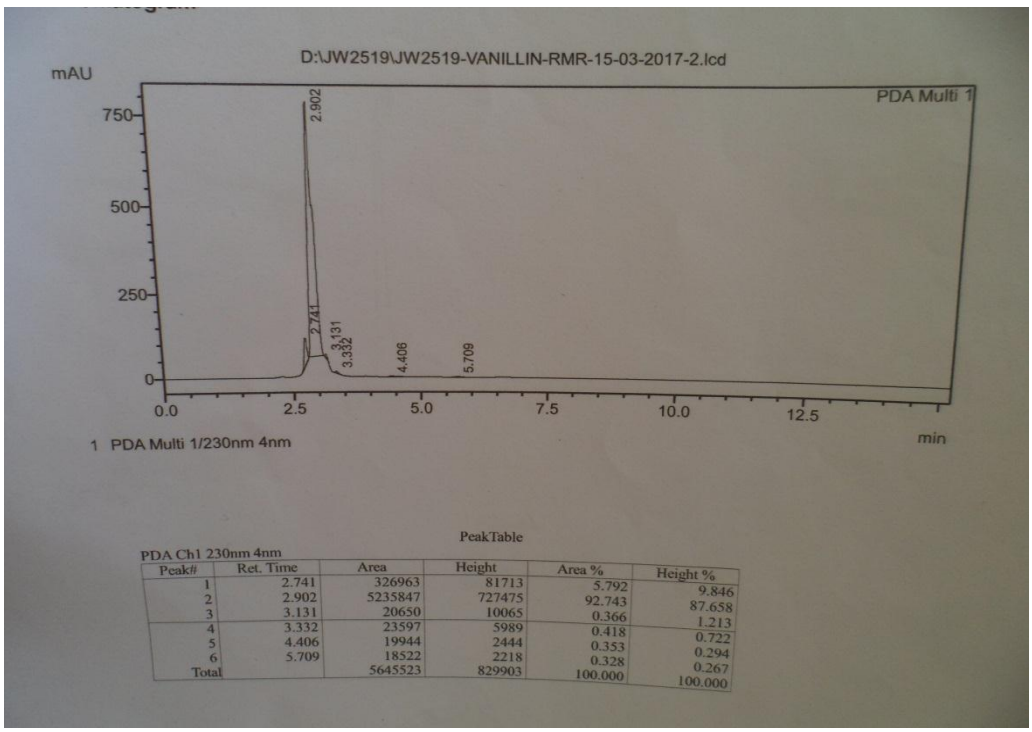
PLATE 3: BIOTRANSFORMATION OF FERULIC ACID BY *PSEUDOMONAS Fluorescens*

1st Day

2nd day

3rd Day

Crude Vanillin



HPLC analysis of vanillin

DISCUSSION

The world market demand for flavors and fragrances, which are widely used in the food and feed as well as cosmetic and pharmaceutical industries, is continuously increasing. In the last decades there has been an increasing consumer trend towards "green" and "eco-friendly" as well as "healthy" processes, which are associated to "bio" or "natural" products. Therefore, natural flavors are favored in the worldwide market despite their considerably higher prices., the industrial attraction for biotechnological production of natural flavors, so-called bioflavors, is increasing constantly in order to replace the traditional chemical processes. In consequence, extensive research studies have been carried out mainly in the field of flavors production from various natural precursors by using several microorganism or single enzymes. Among the flavors, vanillin is, by far, the most important for biotechnological applications. Biotechnological production of vanillin from selected substrates such as ferulic acid could therefore be an interesting alternative.

In the present work the possibility of obtaining vanillin from ferulic acid recovered from wheat bran hydrolyzates by *Pseudomonas fluorescens* has been explored. Meat samples were collected and processed on CFC agar media for the isolation *P.fluorescens*. Based on their biochemical characteristics and pigment production on King's B medium, the isolate was identified as *P.fluorescens*.

Pseudomonas fluorescens isolated from meat samples were explored for biotransformation of ferulic acid to vanillin. Previous reports and research work focused on the use of recombinant *Pseudomonas* strain for accumulating vanillin from ferulic acid.[10] In the present study, an attempt was made to employ wild strain of *P.fluorescens* isolated from meat for the biotransformation reaction resulting in vanillin accumulation.

Initially the research work focused on optimization of biomass production, recovery of ferulic acid and its bioconversion to vanillin. A major drawback using ferulic acid as a substrate for vanillin production is its cost. Therefore an economic ferulic acid recovery from agro-industrial wastes via enzymatic and chemical methods is of interest for several reasons. The most attractive alternative is the possibility of using agro-industrial residues such as wheat bran as low-cost feedstock in the production of value added compounds.[11]

Agro-industrial wastes (or by-products) are "several kinds of biomass materials produced chiefly in food and fibre processing industries", as defined by Bioenergy and Food Security

(BEFS). They are the most abundant source of organic components in the world and hence important natural renewable resources, cheap and readily available. Straw, cereal bran, citrus peelings, cobs, stalks, bagasse are examples of agricultural residues. The use of agro-industrial residues as substrates in biotechnological processes could be a valuable alternative to overcome the high manufacturing costs of industrial fermentations.[12]

Although the bioconversion of ferulic acid to vanillin by several microorganisms was intensely studied, only few papers described the use of ferulic acid obtained from agro-industrial wastes and no work has been reported with wild *Pseudomonas* strains. This was a new attempt in the present investigation. Wheat bran was selected, since wheat is among the most extensively cultivated crops in the world and is a major source of agro-industrial residue. In the present work, wheat bran was used as a substrate for ferulic acid recovery and also a growth substrate for vanillin production. Ferulic acid (FA) is the main phenolic component found in cell walls of monocotyledons, therefore it can be obtained from agro industrial by-products such as corn hulls (31.0 g/kg), maize bran (30 g/kg), sugarbeet (5–10 g/kg), rice endosperm cell wall (9 g/kg), wheat (6.6 g/kg), and barley grains (1.4 g/kg).

Vanillin produced in the fermentation medium was estimated by HPLC and it was run along with standard vanillin. The retention time and the area peaks were used for calculating the concentration of vanillin produced. The peak with retention time 2.902 observed in the fermented sample corresponds to the peak with retention time 2.888 of standard vanillin. The amount of vanillin produced was found to be 12gms/litre. These are in consistent with the results reported by[13] A retention time of 2.76minutes was shown by vanillin produced by *Bacillus subtilis*. The results obtained open the possibility of using the bran hydrolysate both as a growth substrates and as a source of ferulic acid to produce vanillin.

Vanillin is widely used as flavoring in sweet foods like cookies, muffins, cakes, ice creams and soft drinks. Because of its antimicrobial and antioxidant properties[14] vanillin could be used as food preservative, but this potential use is hampered by its strong smell in the inhibitory concentrations required. Synthetic vanillin is used as an intermediate in the production of herbicides, antifoaming agents. It is also widely used as a fragrance ingredient in perfumes and cosmetics and as an intermediate in agrochemicals and pharmaceuticals. Other reports also suggest its use as a nutraceutical due to its reported antimicrobial & antioxidant properties.

The present study therefore indicated the possibility of wheat bran hydrolysate utilization for vanillin flavour production by microorganisms. However the optimization process and evaluation of cost investment are extremely needed. This bioconversion process might therefore be useful for the commercial production of vanillin depending upon the economics of the process as compared with those of chemical synthesis.

CONCLUSION

The biotechnological production of natural vanillin as a feasible alternative to the traditional isolation from *Vanilla* pods is a topic of high interest as demonstrated in the present work. In the search for a novel practicable preparation process for natural vanillin, it has been surprisingly found that this can be obtained economically using bacteria in good yields and concentration by conversion of ferulic acid from agro-industrial waste (wheat bran). The commercial importance of biotechnologically produced vanillin will certainly grow further in the near future due to the growing demand from the consumers side for 'natural' additives for food, feed and cosmetics.

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